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Lack of Buffering by Composites Promotes Shift to More Cariogenic Bacteria

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Abstract

Secondary caries (SC) remains a very important problem with composite restorations. The objectives of this study were to test the acid-buffering ability of several restorative materials and to evaluate whether buffering of the restorative material has an impact on the microbial composition of the biofilm. Disk-shaped specimens of conventional composite, composite with surface prereacted glass-ionomer filler particles (so-called giomer), glass-ionomer cement (GIC), amalgam, and hydroxyapatite (HAp) (control) were exposed to aqueous solutions with pH 4, 5, 6, and 7 and to the medium containing bacteria-produced acids, and pH changes were recorded over several days. Next, material specimens were immersed in bacterial growth medium with pH adjusted to 5. After a 24-h incubation, the extracts were collected and inoculated with a cariogenic (*Streptococcus mutans*) and a noncariogenic (*Streptococcus sanguinis*) species. The bacterial growth was monitored both in a single-species model by spectrophotometry and in a dual-species model by viability quantitative polymerase chain reaction. Amalgam and HAp showed the strongest acid-buffering ability, followed by the GIC and the giomer, while the conventional composite did not exhibit any buffering capacity. Furthermore, due to the lack of acid-buffering abilities, composite was not able to increase the pH of the medium (pH 5), which, in the absence of antibacterial properties, allowed the growth of *S. mutans*, while the growth of *S. sanguinis*, a less aciduric species, was completely inhibited. A similar effect was observed when bacteria were cultured together: there was a higher percentage of *S. mutans* and lower percentage of *S. sanguinis* with the conventional composite than with other materials and HAp. In conclusion, conventional composites lack the ability to increase the local pH, which leads to the outgrowth of more acidogenic/aciduric bacteria and higher cariogenicity of the biofilm. Together with lack of antibacterial properties, lack of buffering may account for the higher susceptibility of composites to SC.

Keywords: amalgam, glass-ionomer cement, *Streptococcus mutans, Streptococcus sanguinis*, quantitative real-time PCR, dental plaque

Introduction

Secondary caries (SC) is one of the most common reasons for the failure of composite restorations, already from the second year after their placement (Opdam et al. 2014). As such, SC seriously compromises the longevity of composite restorations, which was reported to be lower than that of amalgam restorations (Kopperud et al. 2012).

Several prospective clinical studies have indeed shown an SC incidence up to 3.4 times higher with composites than with amalgams (Bernardo et al. 2007; Soncini et al. 2007). Even though patient-related factors such as oral hygiene and dietary habits are considered to play a major role, there are indications that a part of the problem is material based (Nedeljkovic et al. 2015).

Various composites' properties have so far been associated with their higher susceptibility to SC, such as polymerization shrinkage and microleakage, higher plaque receptiveness, the release of bacteria-stimulating compounds, and the lack of antibacterial properties (Nedeljkovic et al. 2015). The ability of a material to neutralize acids produced in dental plaque and to affect the plaque pH, which is crucial for the demineralization/remineralization processes during caries progression, may also play a role. Moreover, according to the ecological plaque hypothesis, local pH changes may lead to compositional shifts in the biofilm (Marsh 1994). Several authors indeed observed higher proportions of cariogenic bacteria next to composite compared with other restorative materials or to unrestored tooth specimens (Svanberg et al. 1990; Thomas et al. 2008). It was suggested that the lack of buffering by composites could lead to the selection of more aciduric bacteria.

Surprisingly, literature on buffering capacity of restorative materials is very scarce and focused mostly on glass-ionomer cements (Nicholson et al. 1999; Mayanagi et al. 2011). Furthermore, the hypothesis that this material property could affect the composition of the overlying biofilm has not been

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Table 1. Materials Used.

Material	Description	Manufacturer
Z100	Hybrid composite Resin: Bis-GMA, TEGDMA Fillers: zirconia/silica	3M ESPE
Beautifil II	Giomer Resin: Bis-GMA, TEGDMA Fillers: S-PRG filler	Shofu
Ketac Fil Plus Aplicap	Conventional glass ionomer cement Liquid: acrylic acid maleic acid copolymer; tartatic acid Powder: glass powder	3M ESPE
Cavex Non Gamma-2	High silver, gamma-2–free amalgam	Cavex

Bis-GMA, bisphenol A-glycidyl methacrylate; S-PRG, surface prereacted glass ionomer; TEGDMA, triethylene glycol dimethacrylate.

tested to date. Therefore, the aim of our in vitro study was 1) to investigate the acid-buffering ability of several restorative materials and 2) to test the effect of this property on the growth and viability of *Streptococcus mutans* and *Streptococcus sanguinis* cultured separately (single-species model) or together (dual-species model) by spectrophotometry and viability quantitative polymerase chain reaction (qPCR).

The null hypotheses were as follows: 1) there is no difference in the acid-buffering ability among different restorative materials, and 2) the buffering ability of the material cannot lead to the imbalance between *S. mutans* and *S. sanguinis* in terms of their growth and viability.

Materials and Methods

Preparation of Specimens

Disk-shaped specimens (2-mm thickness, 7-mm diameter) of a hybrid composite (Z100; 3M ESPE), a so-called giomer (Beautifil II; Shofu), a conventional glass-ionomer cement (Ketac Fil; 3M ESPE), and a dental amalgam (Cavex Non Gamma-2; Cavex) were prepared using polytetrafluoroethylene molds as per the manufacturers' instructions (Table 1). The composites were cured for 40 s at each side with a polywave LED unit (output: 1,400 mW/cm^2) (Bluephase; Ivoclar-Vivadent). Same-size hydroxyapatite (HAp) disks were obtained from Clarkson Chromatography Products Inc.

Screening of Materials' Buffering Ability in Distilled Water

Solutions of distilled water with pH values adjusted to approximately 4, 5, 6, and 7 with HCl (J.T. Baker-Avantor) and NaOH (Merck, Darmstadt, Germany) were prepared. Disks from each material were exposed to 500 µL of these solutions in 48-well plates, after which pH of the solutions was measured at room temperature at 1 h, 24 h, and 48 h with a small electrode (Biotrode; Hamilton) and pH meter (ProfiLine pH 3110; WTW). Solutions without any specimens served as a control. The experiments were carried out in triplicate.

Ability of Materials to Buffer Bacteria-Produced Acids

An overnight culture of *S. mutans* (ATCC 25175) was centrifuged and resuspended in a custom-made buffer-free brain heart infusion (BHI) broth (Appendix Table 1), which was used for all the subsequent experiments. The concentration of the suspension was spectrophotometrically (600 nm) (GeneQuant 100; GE Healthcare) adjusted to 2×10^7 colony-forming units (CFU)/mL. After a 24-h incubation (37°C, 5% CO_2), the bacterial suspension was centrifuged, and the supernatant containing bacteria-produced acids was collected and its pH value was recorded. Specimens of each material (UV-sterilized for 3 h on each side) were then exposed to 250 µL of the supernatant in triplicate in 48-well plates, after which the pH was measured at 24 and 72 h. The supernatant without any specimens served as control. The experiment was repeated 4 independent times (different days, new cultures, and specimens).

Influence of Materials' Buffering Ability on S. mutans *and* S. sanguinis *Growth: A Single-Species Model*

The pH of the custom-made buffer-free BHI was adjusted to 5 by adding lactic acid (Sigma-Aldrich) to imitate the acidic conditions in plaque. Extracts of the materials were prepared by immersing UV-sterilized specimens in 250 µL of this acidified medium for 24 h at 37°C and subsequent filter-sterilization. Overnight cultures of *S. mutans* (ATCC 25175) and *S. sanguinis* (LMG 14657) were centrifuged and resuspended in custom-made BHI, and each species was subsequently added to the extracts in separate 96-well plates to obtain the concentrations of 2×10^7 CFU/mL. During 24-h incubation (37°C, 5% CO_2), the optical density (OD) of these bacterial suspensions was measured spectrophotometrically at 630 nm (Multiskan EX; Thermo Fisher Scientific). As control, both species were also grown in custom-made BHI with pH 7 and 5 without material. This experiment was performed in triplicate and repeated 3 independent times for each species.

Influence of Materials' Buffering Ability on S. mutans *and* S. sanguinis *Viability: A Dual-Species Model*

Extracts of the materials in acidified BHI (pH 5) and overnight cultures of *S. mutans* and *S. sanguinis* were prepared as described earlier (Appendix Fig. 1). For these dual-species experiments, however, both cultures were inoculated into the extracts together in the same 96-well plates, at the same time and same concentration of 2×10^7 CFU/mL. After a 12-h incubation (37°C, 5% CO_2), samples were collected and living *S. mutans* and *S. sanguinis* cells were quantified by viability qPCR with propidium monoazide (PMA) following a previously described protocol (Loozen et al. 2011).

In brief, PMA (Biotium), a photo-reactive DNA-binding dye, was mixed with the dual-species sample. As PMA is cell membrane impermeable, it can only modify the DNA of dead cells, thus preventing its amplification, which results in a selective qPCR quantification of DNA from living cells. After the PMA treatment, bacterial DNA was extracted and purified (QIAamp DNA Mini Kit; Qiagen) and stored at −20°C until qPCR quantification.

qPCR was performed using specific primers and probes (Eurogentec) (Yoshida et al. 2003; Seow et al. 2009) (Appendix Table 2) in a CFX96 Real-Time PCR System (Bio-Rad). Standard curves for absolute quantification were constructed using serial dilutions of complementary DNA (cDNA) plasmids. More specific details on the qPCR protocol can be found in Appendix Table 3 and Appendix Figure 2. These experiments were performed in triplicate 4 independent times.

Statistical Analysis

All statistical analyses were performed by R (version 3.1.1; R Foundation for Statistical Computing). To assess the buffering ability of the materials in distilled water and in bacteria-produced acids, linear mixed-effects models (LMEs) were constructed that estimated the effect of the start pH of the solution, the time and the material on the end pH of the solution, and their interactions. As for the qPCR data, a post hoc Kruskal-Nemenyi test compared the percentages of viable *S. mutans* and *S. sanguinis*, and specific contrasts were calculated to compare the total number of living bacteria between each group and the BHI (pH 5) control. All tests were performed at a significance level of $P = 0.05$.

Results

Buffering Ability of Materials in Distilled Water

Compared with the control solutions (without any material), both HAp and amalgam showed a strong buffering ability by increasing the start pH of all solutions up to around 7 to 8 (Fig. 1a). Also, Ketac Fil was able to increase the pH but only up to 6 (Fig. 1a). In the solution with initial pH 7, the pH even decreased to 6. The buffering ability of Beautifil II was similar to that of Ketac Fil, while the conventional hybrid composite, Z100, did not exhibit any buffering capacity (Fig. 1a, b).

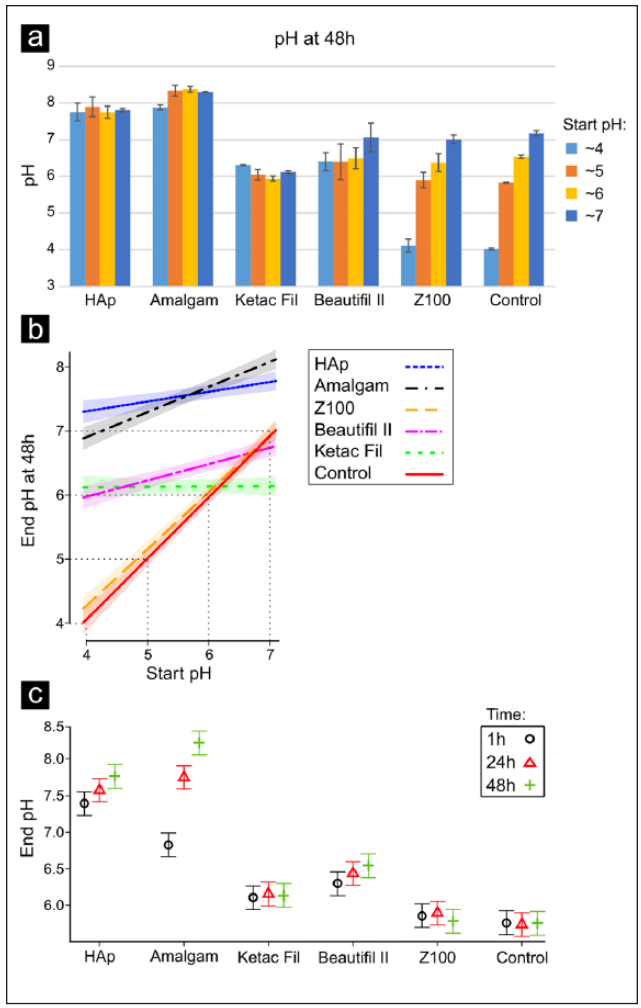

Figure 1. pH values of all water solutions in different groups after 48 h (means and SDs) (**a**) and the effect of material and start pH (**b**) and material and time (**c**) on the end pH of the solution, according to the linear mixed-effects model (LME). (a) Amalgam and hydroxyapatite (HAp) increased the pH of all solutions up to around 8, Ketac Fil and Beautifil II buffered it up to 6 to 7, and the pH of all solutions with Z100 stayed at the same level as the control without any material. (b) The end pH value (y-axis) in function of the start pH value (x-axis) was calculated for each material with the statistical model (LME). Transparent bands around lines denote the 95% confidence intervals (CIs). In the control solutions, the pH did not change, and therefore end pH values corresponded to the same start pH values. The CIs in Z100 are overlapping with those of the control for all the start pH values, since Z100 did not show any buffering ability. In the other groups, however, the end pH values were much higher than the start pH. In the material-time plot (c), symbols denote the end pH values for each measurement time point, as calculated with the LME, and whiskers denote CIs. Only amalgam continued to increase the pH of the solution significantly over time (CI whiskers for 1-h, 24-h, and 48-h values are not overlapping).

The start pH of the solution and the material had a statistically significant effect on the end pH of the solution of distilled water ($P < 0.001$) (Fig. 1b). There was also a significant difference in buffering time between the tested materials. Whereas

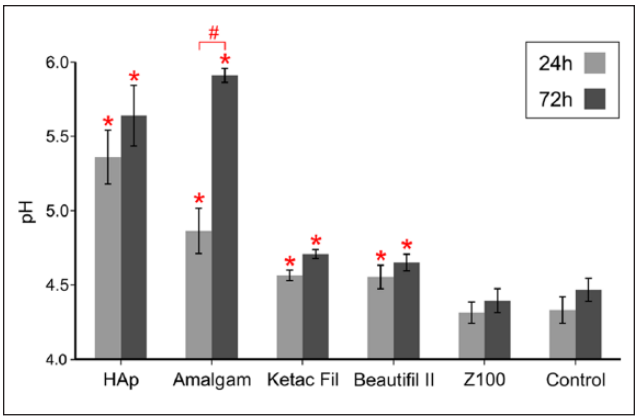

Figure 2. Materials' ability to buffer *Streptococcus mutans*–produced acids (means and SDs from all 4 experiments, $n = 4$). Linear mixed-effects model (LME) showed that after both 24 h and 72 h, all materials except for Z100 significantly increased the pH of the supernatant (asterisks denote values significantly different from the control). Comparison between 24-h and 72-h data was performed by calculating specific contrasts, and only for amalgam, the pH significantly increased over time (# denotes significant difference between 24 h and 72 h). HAp, hydroxyapatite.

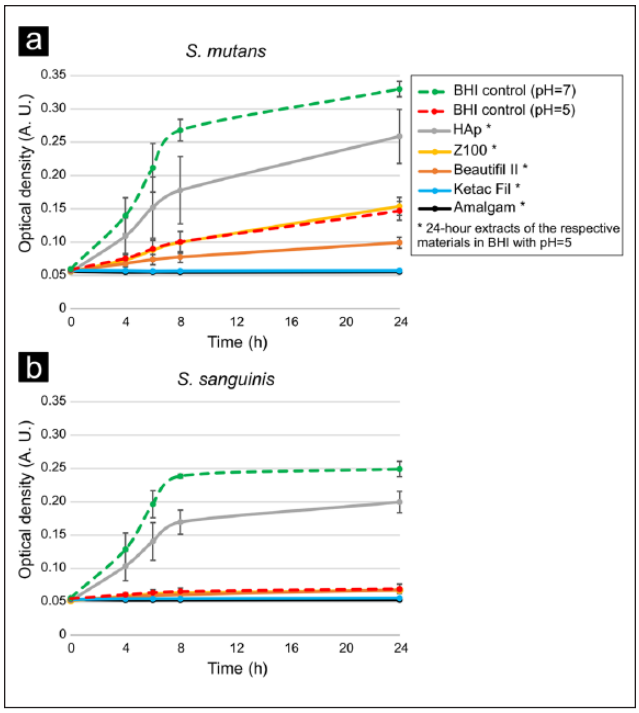

Figure 3. Influence of materials' buffering ability on the growth of *Streptococcus mutans* (**a**) and *Streptococcus sanguinis* (**b**) measured spectrophotometrically (means and SEMs from all 3 experiments are shown, $n = 3$). Dashed lines denote bacterial growth in control brain heart infusion (BHI) media (not incubated with materials) with normal pH (7) and pH adjusted to 5, while full lines denote growth in extracts of respective materials in BHI with start pH 5. Both species grew well in BHI with pH 7. Only *S. mutans* was able to grow well in BHI with pH 5, which demonstrates the higher aciduricity of this bacteria. Amalgam, Ketac Fil, and Beautifil II showed a strong inhibitory effect on the growth of both species, which should mainly be attributed to the release of antibacterial compounds. In the hydroxyapatite (HAp) group, which increased the pH above 6, both species grew well; however, in the Z100 group, in which the start pH of the medium (pH 5) was not changed, only *S. mutans* was able to grow (a), while the growth of *S. sanguinis* was completely inhibited (b).

buffering in HAp, Ketac Fil, and Beautifil II took place already in the first hour, buffering by amalgam was only complete after 48 h (Fig. 1c).

Buffering Ability of Bacteria-Produced Acids

The initial pH of the supernatant containing *S. mutans*–produced acids was 4.33 ± 0.08. Compared with the control (without any material), all materials, except for Z100, significantly increased the pH of this supernatant (Fig. 2). Significant buffering already took place after 24 h. Again, statistical analysis showed a significant effect of the time and the material, as well as their interaction, on the final pH of the supernatant. Furthermore, when the 24-h and 72-h data were compared, only amalgam continued to increase the pH of the solution after 24 h. Finally, acid-buffering abilities of the materials were noticeably lower in the culturing medium with bacteria-produced acids than in distilled water.

S. mutans *and* S. sanguinis *Growth: A Single-Species Model*

The pH values (mean ± SD) of the BHI media with materials' extracts, in which bacteria in both single- and dual-species experiments were cultured, were as follows: HAp = 6.02 ± 0.06, amalgam = 5.77 ± 0.02, Z100 = 4.97 ± 0.04, Beautifil II = 5.14 ± 0.02, and Ketac Fil = 4.86 ± 0.01.

In the single-species model, as expected, only *S. mutans* was able to grow in BHI with pH 5, whereas both species thrived in BHI with pH 7. As for the extracts of the restorative materials in BHI with initial pH 5, only HAp was able to increase the growth of both species, probably due to its strong buffering capacity and the lack of antimicrobial effect. The extracts of amalgam and Ketac Fil completely inhibited the growth of *S. mutans* (Fig. 3a), and a strong inhibition was also observed with Beautifil II. Finally, the growth of *S. mutans* in the extract of Z100 was not inhibited and similar to the growth in BHI with pH 5.

In contrast, the growth of *S. sanguinis* was completely inhibited by the extracts of all the restorative materials, including Z100. *S. sanguinis* was only able to grow in the extract of HAp, which increased the start pH from 5 to above 6 (Fig. 3b).

S. mutans *and* sanguinis *Viability: A Dual-Species Model*

Evaluating the relative amounts of living cells of the 2 species in the control solutions with pH 5, 6, and 7 (Fig. 4a), the proportion of *S. sanguinis* decreased substantially with the decrease in pH of the medium. As for the extracts, there was a

noticeably lower percentage of *S. sanguinis* and a higher percentage of *S. mutans* in the Z100 group compared with other restorative materials. Especially Ketac Fil and HAp favored the viability of *S. sanguinis*, although this was only statistically significant for Ketac Fil.

As for the total amount of living bacteria (Fig. 4b), the viability of the bacteria in the control groups was significantly higher at pH 6 and 7 compared with pH 5. Considering that, it was not surprising to observe a higher viability in the HAp group, which showed a strong buffering ability, and to a certain extent in the Ketac Fil group. Amalgam and Beautifil II, despite their ability to increase the local pH, did not lead to a higher viability of bacteria. Surprisingly, Z100 also led to a higher number of total living bacteria compared with the control with the same pH (pH 5).

Discussion

Although it seems that composites are more susceptible to SC compared with other restorative materials, especially amalgams (Bernardo et al. 2007), the determining material-related factors are not well understood (Nedeljkovic et al. 2015). It has been suggested that the lack of buffering capacity of composites might account for their higher susceptibility to SC by facilitating a compositional shift in the overlying plaque toward higher cariogenicity (Thomas et al. 2008). However, this hypothesis has not been tested to date. In our in vitro study, we demonstrated significant differences in the ability of several restorative materials and HAp to buffer both inorganic and bacteria-produced acids. Furthermore, we showed in a single- and dual-species model that these differences have the potential to cause shifts in plaque toward higher/lower cariogenicity. Therefore, both our null hypotheses were rejected.

S. mutans is a well-known cariogenic species (Loesche 1986), while *S. sanguinis* was selected as a noncariogenic species because it interacts antagonistically with *S. mutans* by producing peroxides (Kreth et al. 2005, 2008), and it is more prevalent in caries-free individuals than in individuals with caries (Giacaman et al. 2015). Thus, it has been postulated that an imbalance between these 2 species may result in a higher or lower cariogenicity of the plaque.

As a control material, we used HAp disks, which were able to buffer all aqueous acid solutions. The lower the starting pH, the higher pH increase was observed, which is a result of a higher dissolution/demineralization of HAp at lower pH values. As such, the more PO_4^{3-} and OH^- are released, the more H^+ will be neutralized, thereby increasing the pH of the solution until the saturation point is reached (Fejerskov and Kidd 2008).

Amalgam also showed a strong acid-buffering ability, which should be attributed to the release of corrosion products. Zinc is a strong reducing agent, and tin and copper oxides are amphoteric compounds that react as a base in acidic conditions (Sutow et al. 1991; Sanna et al. 2002). It is already known that amalgam corrosion products can seal the interfacial gap and prevent microleakage, thereby improving amalgam's resistance to SC (Ben-Amar et al. 1995). However, the acid-buffering

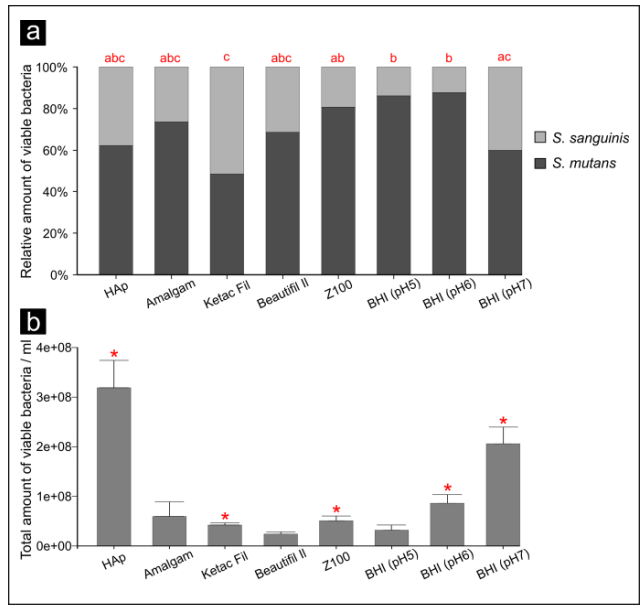

Figure 4. Relative amounts of viable *Streptococcus mutans* and *Streptococcus sanguinis* (**a**) and total amount of viable bacteria (#/ml) (**b**) in samples of dual-species cultures, quantified by viability quantitative polymerase chain reaction (n = 4). (a) In control samples (brain heart infusion [BHI] [pH 5], BHI [pH 6], and BHI [pH 7]), the proportions of the 2 species depended on the pH of the medium: the percentage of *S. sanguinis* dropped significantly with the decrease in pH. Among all tested materials, the highest percentage of *S. mutans* and the lowest percentage of *S. sanguinis* were observed with Z100. The proportion of bacteria significantly differed from Ketac Fil (letters denote different statistical groups). (**b**) The total amount of living bacteria (means and SEMs are shown) was also pH dependent (BHI [pH 5], BHI [pH 6], and BHI [pH 7] controls) (asterisks denote values significantly different from BHI [pH 5] control). Hydroxyapatite (HAp) increased the pH of the medium and thereby the number of living cells, as well as Ketac Fil. Surprisingly, even with Z100, the number of living bacteria was higher than in the control with the same pH (pH 5).

ability of amalgam demonstrated in our study should be considered an additional cariostatic property of this restorative material. Finally, we confirmed the already known antibacterial effect of the amalgam (Beyth et al. 2007) in both the single- and dual-species model, which probably stems from the release of metal ions such as mercury, copper, and zinc (Morrier et al. 1998).

Glass-ionomer cements (GICs) are well known for their cariostatic behavior, which is based not only on their antibacterial effect (Duque et al. 2005; Tegginmani et al. 2013) but also on the inhibition of bacterial acid production (Nakajo et al. 2009) due to the fluoride release. Both of these properties will contribute to a lower acidity of the plaque around GIC compared with resin composites (Mayanagi et al. 2014). In addition, we demonstrated that Ketac Fil can directly buffer solutions of inorganic as well as the bacteria-produced acids; however, this effect depended largely on the composition and the start pH of the medium. Our findings are consistent with those of Nicholson et al. (1999, 2000), who observed the same effect of several GICs in an aqueous lactic acid solution. It was also observed that the extract of Ketac Fil, unlike those of the

other tested materials, behaved as a real chemical buffer, changing the pH of all starting solutions toward a stable value of 6. This behavior can be explained by the release of unreacted acrylic (or other organic acid) and its calcium salt, a weak acid, and its conjugate base, which constitute a typical chemical buffer (Nicholson et al. 2000). Nevertheless, the buffering abilities of all the materials were noticeably lower in the BHI medium than in distilled water, probably due to the presence of various proteins, oligopeptides, and amino acids with buffering abilities (zwitterions) in the BHI medium.

Z100, a conventional hybrid composite, was the only tested material that did not demonstrate any acid-buffering ability. Therefore, apart from an already documented lack of antibacterial properties (Boeckh et al. 2002), conventional composites are also lacking the potential to decrease the acidity of the plaque, a cariostatic property exhibited by other tested materials. In the microbiological part of our study, we demonstrated that the inability of Z100 to increase the local pH can lead to an outgrowth of more acidogenic/aciduric bacteria. As the pH of the medium incubated with Z100 stayed as low as 5, only *S. mutans* was able to grow, while the growth of a much less aciduric species, *S. sanguinis*, was completely inhibited. Furthermore, when the 2 species were cultured together in a dual-species model, we could observe the highest percentage of viable *S. mutans* and the lowest percentage of *S. sanguinis* in the extract of Z100, even though these ratios were only significantly different from Ketac Fil, which had the most desirable bacterial distribution. These data produce a strong evidence for the hypothesis that the lack of buffering abilities of a restorative material can facilitate compositional shifts in plaque toward higher cariogenicity by maintaining conditions that are hostile for beneficial but sustainable for most aciduric/cariogenic bacteria. As such, this can to a certain extent explain the higher proportion of mutans streptococci in plaque growing on composites compared with amalgams, GICs, and even HAp (Svanberg et al. 1990; Thomas et al. 2008).

Surprisingly, the total number of viable bacteria in the Z100 group was significantly higher than in the medium control with pH 5 (Fig. 4b). This can signify that composites may release compounds that increase the viability of oral bacteria, as has been suggested before (Kawai et al. 1988; Hansel et al. 1998), but more research is warranted to identify which compounds might be responsible for this effect.

In this study, we also included a so-called giomer, which is basically a traditional methacrylate-based composite with surface prereacted glass-ionomer (S-PRG) fillers, since it could be hypothesized that modified composites with better buffering capacities should be less prone to SC. Giomers were developed in an attempt to give composites the cariostatic properties of GICs. In our study, we observed that Beautifil II was indeed capable of increasing the pH of the solutions up to neutral (6 to 7), which is in agreement with the results of Kaga et al. (2014), who reported a very similar effect of BeautiSealant, an S-PRG–containing fissure sealant. This implies that the buffering ability should be attributed to S-PRG fillers. In addition, the observed bacteriostatic effect of Beautifil II is in agreement with previous findings (Saku et al. 2010; Tarasingh et al. 2015). Extracts of Beautifil II also tended to result in a more favorable ratio of *S. mutans*/*S. sanguinis* compared with the conventional composite.

The high susceptibility of composites to SC has long been an issue of a scientific debate, and great efforts were made to design a composite with cariostatic properties, mostly by the incorporation of antibacterial compounds. However, we have demonstrated in this study that the microbial composition is affected not only by the antibacterial activity of the material, but also by its ability to counteract the acidification of plaque.

Considering the fact that SC develops over months, future research should focus on investigating whether buffering capacity of the restorative materials is affected by aging and by time. As a 2-species model considerably simplifies the complex bacterial community of dental plaque, a multispecies setup will surely give more clinically relevant information about microbial shifts caused by certain materials' properties.

To conclude, restorative materials differ markedly in their capacities to buffer acids, with the conventional composite showing no buffering ability. Buffering capacity directly influences the demineralization process of the adjacent tooth tissue, but we also demonstrated that the inability of composite to increase the local pH facilitates the outgrowth of more aciduric and cariogenic bacteria. This bacterial shift toward more cariogenic species, along with the lack of antibacterial properties, polymerization shrinkage, and subsequent microleakage, will contribute to the higher susceptibility of composites to SC. Buffering ability is an important material property that should be taken into account while designing more caries-resistant composites.

Author Contributions

I. Nedeljkovic, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; J. De Munck, contributed to design, data analysis, and interpretation, critically revised the manuscript; B. Van Meerbeek, contributed to conception, data analysis, and interpretation, critically revised the manuscript; W. Teughels, contributed to conception, design, and data interpretation, critically revised the manuscript; V. Slomka, contributed to data interpretation, critically revised the manuscript; K.L. Van Landuyt, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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